Addendum A: Stepwise approach to identify causative organism or non-infective cause

A stepwise approach was employed to identify the causative organisms of IE and to minimise the incidence of BCNIE. At least three sets of blood cultures per patient were collected using an aseptic technique. Each set was drawn from a different peripheral site and included a BacT/ALERT® FA Plus (aerobic) (bioMérieux, Marcy l'Étoile, France) and BacT/ALERT® FN Plus (anaerobic) bottle. Additional blood cultures were obtained if clinical features of infection persisted. The blood cultures were submitted to the Microbiology laboratory of the National Health Laboratory Service (NHLS) at Tygerberg Hospital, and incubated in the BacT/ALERT® 3D automated microbial detection system for five days. Once the instrument detected growth in the bottles and signalled them as positive, they were removed, a Gram stain performed and the clinician immediately phoned and informed of the result. Depending on the organism observed on Gram stain, the blood culture broth was sub-cultured onto appropriate solid culture media such as blood agar, chocolate agar and MacConkey agar (prepared in-house), followed by incubation at the required temperature and atmosphere to optimise growth. The agar plates were examined after 18-24 hours of incubation and isolates identified using manual and/or automated biochemical assays; if these methods failed, isolates were referred for mass spectrometry (VITEK® MS, bioMérieux, Marcy l'Étoile, France). Antimicrobial susceptibility testing (AST) was performed by disk diffusion, automated broth dilution (VITEK® 2, bioMérieux, Marcy l'Étoile, France) and/or Etest® (bioMérieux, Marcy l'Étoile, France). AST results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) criteria. If no organism was isolated after five days using standard culture techniques, patients were defined as having BCNIE. Further testing performed on these patients are summarised in Figure 1 and included:

- Serology: Indirect immunofluorescence assays (IFA) for detection of antibodies to *Bartonella henselae* and *B. quintana* (Bartonella IFA IgM and IgG kits, FOCUS Diagnostics, Cypress, CA, USA), and *C. burnetii* (Q Fever IgM and IgG kits, FOCUS Diagnostics, Cypress, CA, USA). Enzyme-linked immunosorbent assays (ELISA) were performed to detect IgM and IgG antibodies to *Brucella* species (MASTAZYME BRUCELLA kit, MAST DIAGNOSTICA, Reinfeld, Germany), *Legionella pneumophila* (EUROIMMUN, Lübeck, Germany) and *Mycoplasma pneumoniae* (EUROIMMUN, Lübeck, Germany).
- Auto-antibody testing: Antinuclear antibodies (ANA) were detected with the Kallestad® HEp-2 Cell Line Substrate and Kallestad Mouse Stomach/Kidney Test kits (Bio-Rad Laboratories Inc, Redmond, WA, USA), and anti-cardiolipin antibodies (ACLA) with the QUANTA Lite PR3 SC and MPO SC ELISA kits (Inova Diagnostics, San Diego, CA, USA).

- Molecular testing on negative blood cultures: DNA was extracted from 200 µl of blood culture broth using the tissue protocol of the Qiagen QIAamp DNA Mini Kit, following benzyl alcohol extraction, as previously described. (1) Bacterial 16S rRNA PCR amplification was performed using primers BAK11w and BAK2 (2) and KAPA2G Robust HotStart ReadyMix (Kapa Biosystems, South Africa) according to manufacturer's instructions, with an annealing temperature of 55°C for 30 cycles. Fungal ITS2 amplification was performed using primers ITS1 and ITS4 (3) and KAPA Taq ReadyMix (Kapa Biosystems) in a touchdown PCR with annealing temperatures of 56°C for 10 cycles and 54°C for 30 cycles. Amplicons were sequenced on the ABI 3500XL genetic analyser at Inqaba Biotec (South Africa). Bacterial and fungal identification was based on >99% sequence alignment to published sequences available in the National Center for Biotechnology Information's Genbank database.
- Mycobacterial blood cultures: BACTEC™ Myco/F Lytic Culture vials (Becton Dickinson, Sparks, MD, USA) were collected for the isolation of *Mycobacterium tuberculosis* (MTB) and non-tuberculous Mycobacteria. The bottles were incubated in an automated continuous monitoring BACTEC 9120 instrument for 42 days. Work-up of positive cultures is not included since all cultures were negative.

If surgery was performed, heart valve tissue was submitted for:

- Bacterial and fungal culture
- Broad range PCR and sequencing of 16S rRNA for bacteria and ITS2 for fungi
- Histopathologic examination for detection of bacteria and fungi, as well as histopathological features of IE.

References:

1. Christensen *et al*. (2003). Rapid Identification of Bacteria from Positive Blood Cultures by Terminal Restriction Fragment Length Polymorphism Profile Analysis of the 16S rRNA Gene. *J. Clin. Microbiol.* 41:3790-3800
2. Bosshard *et al*., 2006. 16S rRNA Gene Sequencing verses the API 20 NE system and the VITEK 2 ID-GNB Card for Identification of Non-fermenting Gram-Negative Bacteria in the Clinical Laboratory. *J. Clin. Microbiol*. 44: 1359-1366
3. White *et al*., (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: a guide to methods and applications. (Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds). Academic Press, New York, USA: 315–322